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Phylogeny and Taxonomy of Freshwater *Bangia* (Bangiales, Rhodophyta) in Japan

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Two samples of the red alga *Bangia* from two rivers, the Amahata-gawa and Ôi-gawa, Japan, were phylogenetically analyzed. The sequences of their nuclear 18S rRNA gene and their chloroplast *rbcL* gene were similar or identical to a freshwater *Bangia*, *B. atropurpurea* (Roth) C. Agardh of North American and Europe. The freshwater *Bangia* comprised a distinct clade from marine *Bangia* in both 18S rRNA and *rbcL* phylogenetic trees. This result showed that the freshwater *Bangia* found in Japan share a common ancestor with the North American and European freshwater *Bangia*. Like the North American and European freshwater *Bangia*, it is appropriate to classify the Japanese freshwater *Bangia* as *B. atropurpurea*.

Key words: 18S rRNA gene, *Bangia atropurpurea*, freshwater *Bangia*, phylogenetic tree, *rbcL* gene.

The red algal genus *Bangia* is widespread along the marine coasts of the world (e.g., Schneider and Searles 1991, Sheath and Cole 1984, Silva et al. 1996, Womersley 1994, Yoshida 1998). Its existence in freshwater in North America, Europe, and East Asia has also been reported (e.g., Okada 1944, Roth 1806, Sheath and Cole 1980).

Roth (1806) originally described freshwater *Bangia* as *Conferva atropurpurea* Roth. Later Agardh (1824) classified it as *Bangia atropurpurea*. Dellwyn (1802–1809) originally described marine *Bangia* as *Conferva fuscopurpurea* Dillwyn. Later Lyngbye (1819) classified it as *B. fuscopurpurea* (Dillwyn) Lyngbye. One distinguishing character between them is whether they live in freshwater or in marine environments. Several researchers (Geesink 1973, Reed 1980, Sheath and Cole 1980), however, have shown that *Bangia* can acclimatize to a wide range of salinities, and Geesink (1973) concluded that the freshwater *B. atropurpurea* and the marine *B. fuscopurpurea* are conspecific, and suggested classifying them both as *B. atropurpurea*. On the other hand, more recently, analyses of RuBisCo spacer, *rbcL* gene and 18S rRNA gene sequences indicated that while the North American and European freshwater specimens are nearly identical, they are considerably different from marine specimens (Müller et al. 1998,

2003).

Müller et al. (2003) re-examined the systematics of *Bangia* based on karyological and molecular phylogenetic analyses, and demonstrated that the freshwater *Bangia* from North America and Europe share distinctive chromosome morphologies, and are positioned on a well-supported branch in phylogenetic trees. They proposed that *B. atropurpurea* should be re-recognized as a distinct species from *B. fuscopurpurea.*

Freshwater *Bangia* in Japan was first reported by Okada (1944). He found it on rocks and dead trees in a freshwater tributary stream of the Amahata-gawa river in Yamanashi Prefecture and identified it as *B. atropurpurea.* There have been no reports of this alga in any other river or lake in Japan until now. Moreover, no molecular phylogenetic studies have been done on this alga in Japan. Recently, we also found the freshwater *Bangia* in the Ôi-gawa river in Shizuoka Prefecture. We collected from both of these rivers and performed molecular phylogenetic analyses based on 18S rRNA gene and *rbcL* gene sequences. We discuss their phylogeny, taxonomy and biogeography.

Materials and Methods

Two samples of freshwater *Bangia* were collected from two rivers in Japan (Table 1). The *Bangia* collected from a tributary of the Amahata-gawa river in Yamanashi Prefecture was found growing on rocks at the bottom of the sunny side of a cliff in Okusawa-dani which is about 650 m above sea level. The *Bangia* collected from the Higashikawauchi-gawa river, a tributary of the Ôi-gawa in Shizuoka Prefecture, was found on rocks at the bottom of the sunny side of a concrete dam at about 800 m above sea level.

Total DNA was extracted from the *Bangia* samples using the CTAB miniprep method of Doyle and Doyle (1990). A portion of the nuclear 18S rRNA gene was amplified by a polymerase chain reaction (PCR) using the primer pair SR1 and SR12 (Table 2). The PCR products were purified with an Ultra Clean 15 kit (Mo Bio Laboratories, Solana Beach, CA, USA) after agarose gel electrophoresis. The purified PCR products were sequenced using a Thermo Sequence cycle sequencing kit (USB, Cleveland, OH, USA) and an A. L. F. 2 autosequencer (Amersham Biosciences, Piscataway, NJ, USA). Sequencing reactions for the 18S rRNA gene were performed using primers SR1 and SR12 and eleven internal primers: SR2, SR3, SR4, SR5, SR7, SR9, 18S-1R, 18S-2R, 18S-5M, 18S-10M, and 18S-13M.

The nucleotide sequences of portions of the chloroplast Rubisco large subunit (*rbcL*) gene were also determined. The primer pair rbcL-Rh1 and rbcS1 was used for the PCR amplification, and three internal primers (rbcL-Rh2, rbcL-Rh3, and rbcL-Rh4) were used for the sequencing reactions with the primers rbcL-Rh1 and rbcL-Rh2 (Table 2).

Table 1. Collections of freshwater *Bangia* from Japan used in molecular analyses based on the nuclear 18S rRNA gene and chloroplast *rbcL* gene sequences

Symbol	Location	Accession number	
		18S rRNA	*rbcL*
SH	Higashikawauchi-gawa, Ôi-gawa, Ikawa, Shizuoka City, Shizuoka Pref., J. Suzawa, 20 March 2001	AB114638	AB114640
YA	Okusawa-dani, Amahata-gawa, Hayakawa Town, Yamanashi Pref., T. Hanyuda, 20 April 2001	AB114639	AB114641

Table 2. Primers used to amplify and sequences of 18S rRNA and *rbcL* genes

Primer	Gene	Sequence (5′ to 3′)	Reference
SR1	18S	TAC CTG GTT GAT CCT GCC AG	Nakayama & al. 1996
SR2	18S	CAT TCA AAT TTC TGC CCT ATC	Nakayama & al. 1996
SR3	18S	AGG CTC CCT GTC CGG AAT C	Nakayama & al. 1996
SR4	18S	AGC CGC GGT AAT TCC AGC T	Nakayama & al. 1996
SR5	18S	ACT ACG ACT TTT TAA CTG C	Nakayama & al. 1996
SR7	18S	TCC TTG GGC AAA TGC TTT CG	Nakayama & al. 1996
SR9	18S	CGC TCC ACC AAC TAA GAA C	Nakayama & al. 1996
SR12	18S	CCT TCC GCA GGT TCA CCT AC	Nakayama & al. 1996
18S-1R	18S	TTA GAT GTT CTG GGC CGC AC	in this study
18S-2R	18S	CAA CYG TCC CTM TTA ATC ATT AC	in this study
18S-5M	18S	AGC CCC ATC ACG ATG CAG TT	Hanyuda & al. 2002
18S-10M	18S	GAC GGT CGG GGG CAT TCG TA	in this study
18S-13M	18S	GCT TAA TTT GAC TCA ACA CGG	in this study
rbcL-Rh1	*rbcL*	AAG TGA ACG TTA CGA ATC TGG	in this study
rbcL-Rh2	*rbcL*	GAT ATW GAT TTA TTY GAA GAA GG	in this study
rbcL-Rh3	*rbcL*	TTA AYT CTC ARC CDT TYA TGC G	in this study
rbcL-Rh4	*rbcL*	TGY AAR TGG ATG MGW ATG GC	in this study
rbcS1	*rbcL*	AAA AGY YCC TTG TGT TAR TCT CAC	in this study

The 18S rRNA gene sequences determined in the current study were aligned by eye referring to the sequence alignments of *Bangia* (Müller et al. 1998) stored in the European Ribosomal RNA database (http://oberon.fvms.ugent.be:8080/rRNA/), which had been aligned according to a secondary structure model. Group IC1 introns, which are widely present in the 18S rRNA genes of Bangiales (Müller et al. 2001), were removed for the alignment. Previously published sequences of *Bangia* (Müller et al. 1998, 2003, Shimomura et al. 1999), *Porphyra* (Ragan et al. 1994), and the outgroup taxa, *Erythrotrichia carnea* (Dillwyn) J. Agardh and *Erythrocladia* sp. (Ragan et al. 1994) were obtained from the European Ribosomal RNA database or the DDBJ (DNA Data Bank of Japan), and used for the analysis described below.

The *rbcL* gene sequences determined in this study were also aligned by eye with previously published sequences of *Bangia* (Müller et al. 1998, 2003) and the outgroup taxa, *Erythrotrichia carnea* and *Erythrocladia* sp. (Rintoul et al. 1999). The alignment was easy to do because there were no insertions or deletions.

Maximum likelihood (ML) and maximum parsimony (MP) analyses were carried out using the software PAUP 4.0b10 (Swofford 2002). The data sets were tested with ModelTest 3.06 to find the model of evolution that best fitted the data (Posada and Crandall 1998). ML analyses based on the 18S rRNA gene and *rbcL* gene sequences were performed using tree bisection-reconnection branch swapping of the heuristic search with random sequence addition (100 replicates), collapse option, and MULPARS option. For the 18S rRNA gene, as the Tamura-Nei model (Tamura and Nei 1993), incorporating invariable sites (0.4100) and the gamma distribution parameter (0.5180), was the best-fit evolutionary model, it was used for the analysis. For the *rbcL* gene, the General Time Reversible (GTR) model (Rodríguez et al. 1990), incorporating invariable sites (0.4630) and the gamma distribution parameter (1.9423), was

selected. MP trees were also constructed using the Branch-and-bound search option with furthest sequence addition (18S rRNA) or as-is sequence addition (*rbcL*). Bootstrap probabilities were obtained from 100 replicates using the heuristic search option with as-is sequence addition and nearest-neighbor interchange branch swapping in ML analysis, and from 5000 replicates using the heuristic search option with simple sequence addition and nearest-neighbor interchange branch swapping in MP analysis.

Results

All sequence data generated in this study were submitted to DDBJ (DNA Data Bank of Japan, Table 1). There were two nucleotide substitutions in the 18S rRNA gene between the two Japanese collections (SH and YA). 18S rRNA gene sequences of the two Japanese collections were similar to those of the North American and the European *B. atropurpurea*. Sequence differences ranged from 0-4 nucleotide substitutions. The *rbcL* gene sequence of the collection from the Oigawa river (SH) was identical to those of *B. atropurpurea* from the Great Lakes in North America and from Italy, and differed from the other Japanese collection by one nucleotide. Sequence differences among freshwater collections from Japan, North America, and Europe ranged from 0–20 nucleotide substitutions.

The ML tree based on the 18S rRNA gene sequences is shown in Fig. 1. The *Bangia* and *Porphyra* species are in either clade 1 or

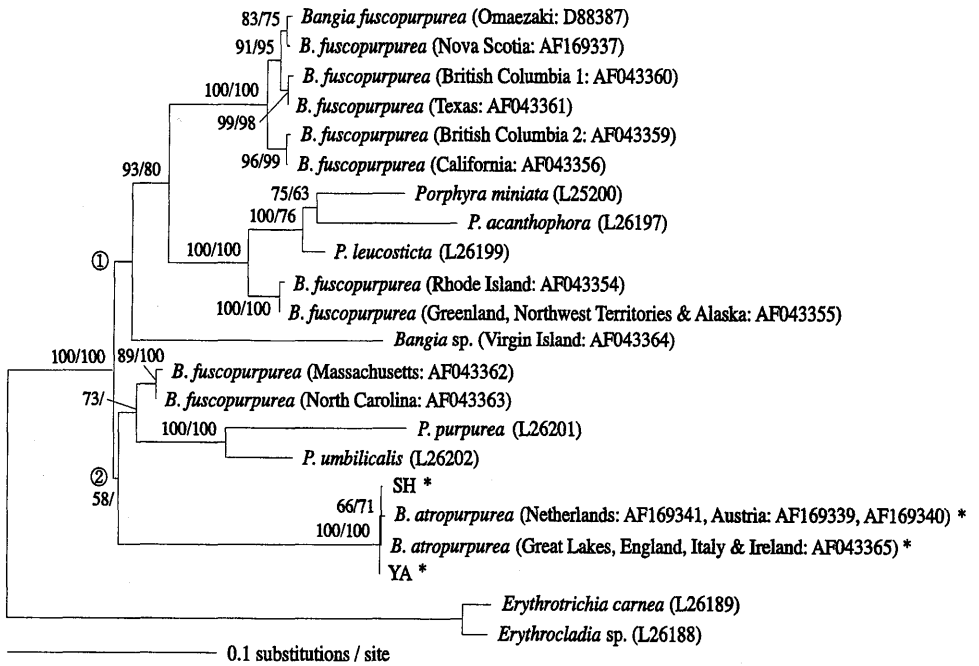

Fig. 1. Maximum likelihood tree of 22 operational taxonomic units (OTUs) based on 18S rRNA gene sequences (log likelihood = –8104.62). Numbers associated with nodes indicate bootstrap values for maximum likelihood (left) and maximum parsimony (right), respectively. Nodes with bootstrap values of less than 50 % are not labeled. Names and numbers in the parentheses indicate localities and accession numbers. Numbers ① and ② indicate major clades. Asterisks indicate freshwater collections.

clade 2. Clade 1 has three subclades: one with only *Bangia* sp. from Virgin Island, another with *Bangia fuscopurpurea* from North America and Japan, and one with *B. fuscopurpurea* from the Northern Hemisphere and three *Porphyra* species. The collections from the two Japanese rivers (SH and YA) are in clade 2 and form a well-supported subclade (100/100 %) with *B. atropurpurea* from the Northern Hemisphere. The phylogenetic relationship within this subclade is unclear. The MP analysis yielded three most parsimonious trees of 1163 steps, with a consistency index of 0.645 and a retention index of 0.799 (trees not shown). The phylogenetic relationships in these trees were identical to those of the ML tree, except that *Bangia* sp. (Virgin Island) was located at the most basal position in the in-group, and a subclade of clade 2 had *P. purpurea* (Roth) C. Agardh and *P. umbilicalis* (L.) Kütz. forming a clade with a subclade of freshwater *Bangia*.

The ML tree based on the *rbcL* gene sequences showed that all the *Bangia* samples are in clades 1 and 2, except for *Bangia* sp. from Virgin Island (Fig. 2). Clade 1 is composed of *B. fuscopurpurea*, and clade 2 is composed of the freshwater *Bangia* from the Northern Hemisphere (North America, Europe, and Japan), supported by high bootstrap values (100/100 %). In clade 2, the position of *B. atropurpurea* from Ireland and Austria is well supported (100/100 %), but the relationship among the other freshwater populations is ambiguous. Six MP trees (765 steps, consistency index = 0.663, and retention index = 0.730) were generated by the MP analysis (trees not shown). The overall phylogenetic relationships in these trees are similar to those of the ML tree, and clade 2 was present in all of the MP trees. Clade 1 was absent in the MP trees and *B. fuscopurpurea* from Massachusetts, Rhode

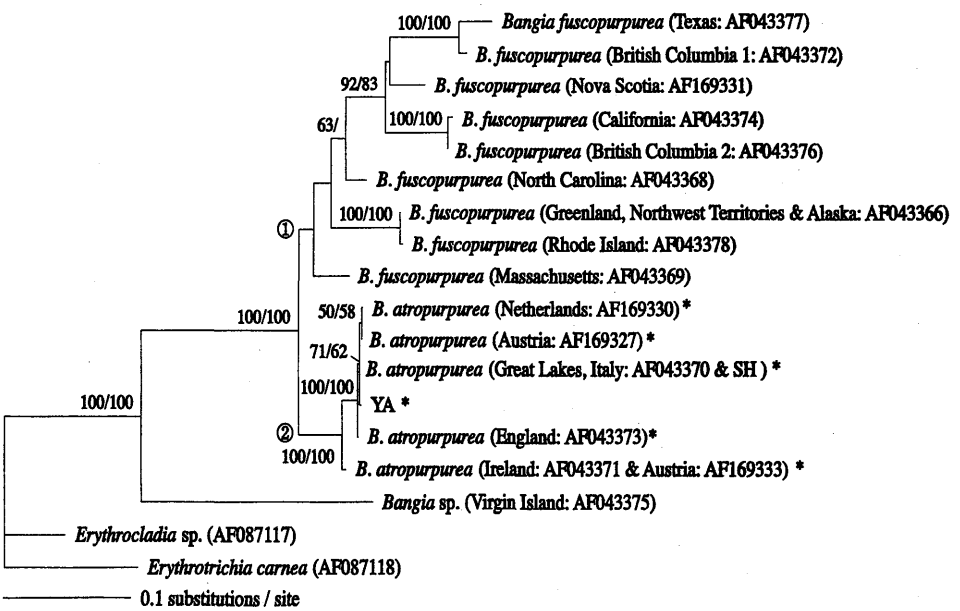

Fig. 2. Maximum likelihood tree of 18 operational taxonomic units (OTUs) based on *rbcL* gene sequences (log likelihood = –5014.67). Numbers associated with nodes, names and numbers in the parentheses, numbers ① and ②, and asterisks follow Fig. 1.

Island, North Carolina, Greenland, Northwest Territories, and Alaska formed a monophyletic group with clade 2.

Discussion

As mentioned above, two molecular sequence analyses showed that two Japanese collections of freshwater *Bangia* are similar or identical to *B. atropurpurea* from North American and European freshwaters, and that these freshwater *Bangia* formed a distinct lineage in all ML and MP trees. These results show that all freshwater *Bangia* referred to in this study have a common ancestor, and may suggest that the adaptation of *Bangia* to freshwater environment happened once only at a common ancestor. Based on distinctive chromosome morphology and the monophyletic relationship of the North American and European freshwater *Bangia*, Müller et al. (2003) proposed that freshwater *Bangia* should be re-recognized as the distinct species, *B. atropurpurea*. Therefore, based on the results of the current study, it is appropriate to classify the Japanese freshwater *Bangia* as *B. atropurpurea* together with the North American and the European freshwater *Bangia*, though the karyotypes of the Japanese freshwater *Bangia*, and of the North American and the European *B. atropurpurea* have not yet compared.

Although the existence of freshwater *Bangia* in many localities in the Northern Hemisphere has been reported, most of these localities are far apart. If continental drift played a role in such isolated distributions across different continents, the genetic variations among the populations would be large. However, the sequence divergences among the freshwater collections examined in this study are very small, especially in the 18S rRNA gene. This suggests that the distribution of freshwater *Bangia* throughout the world is a result of geologically recent long-distance dispersal.

In the *rbcL* gene tree, the *B. atropurpurea* sample from Ireland and Austria are located in the most basal position in the clade 2 composed of all the freshwater collections examined, and the position is well supported. The two Japanese populations are more derived than these two European populations, suggesting that the Japanese freshwater *Bangia* originated from a freshwater source, not from a marine source.

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羽生田岳昭[a]，洲澤　譲[b]，新井章吾[c]，植田邦彦[d]，熊野　茂[e]：**日本産淡水紅藻 *Bangia* (Bangiales, Rhodophyta) の系統と分類**

日本の2河川，雨畑川と大井川から採集された紅藻 *Bangia* の系統解析を行った．これらの核の18S rRNA 遺伝子および葉緑体の *rbcL* 遺伝子の塩基配列は，北米やヨーロッパの淡水産 *Bangia* (*B. atropurpurea* (Roth) C. Agardh) のそれと同一かまたは類似していた．淡水産 *Bangia* は，18S rRNA 遺伝子および *rbcL* 遺伝子のどちらの系統樹上においても明瞭な単系統群（クレード）を形成した．日本で見つかった淡水産 *Bangia* が，北米やヨーロッパの淡水産 *Bangia* と共通祖先を共有することが示された．北米やヨーロッパの淡水産 *Bangia* のように，日本の淡水産 *Bangia* を *B. atropurpurea* に分類するのが適当である．

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